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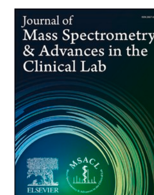

Research Article

Liquid chromatography-mass spectrometric method for the simultaneous analysis of branched-chain amino acids and their ketoacids from dried blood spot as secondary analytes for the detection of maple syrup urine disease

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A R T I C L E I N F O

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A B S T R A C T

Background: Maple syrup urine disease (MSUD) is an aminoacidopathy caused by a defective branched-chain alpha-ketoacid dehydrogenase complex, leading to the accumulation of branched-chain amino acids (BCAAs) and their respective keto acids (BCKAs). A comprehensive test was developed to measure BCAAs and BCKAs using LC-MS from dried blood spot (DBS) samples for the diagnosis and prevention of MSUD in newborns and infants.

Methods: Analytes were extracted from DBS using a methanol:0.1 % v/v formic acid solution (75:25) containing internal standards and analyzed on a Luna PFP column (150 mm × 4.6 mm, 3 μm) at a flow rate of 0.3 mL/min. The method was validated for linearity, accuracy, precision, recovery, carry-over, matrix effect, hematocrit, blood volume, and punch position effects. Biomarker stability in the matrix and stock solution was assessed. Correlation with the plasma method was determined using Pearson's correlation coefficient and Bland-Altman analysis. The method established reference ranges for the Udupi district population in South India.

Results: The method demonstrated linearity ($r^2 > 0.99$), with a lower limit of detection at 2 μM (BCAA) and 1 μM (BCKA), and acceptable recovery of QC samples. Hematocrit, blood volume, punch position, and storage condition effects were within acceptable limits. Correlation and Bland-Altman analysis showed strong interconvertibility between plasma and DBS assays. Reference ranges for leucine, isoleucine, valine, KIC, KIV, and KMV were established.

Conclusion: The developed DBS method, requiring no derivatization and involving simple sample preparation with short run times, is a cost-effective and reliable approach for the confirmatory diagnosis of MSUD.

Abbreviations: AGREE, Analytical GREEnness; BCAA, Branched-chain amino acid; BCKA, Branched-chain keto acids; BCKAD, Branched-chain alpha-keto acid dehydrogenase; CE, Collision Energy; CV, Coefficient of variation; DBS, Dried blood spot; EDTA, Ethylene diamine tetra acetic acid; ESI, Electrospray ionization; HCT, Hematocrit effect; HQC, High Quality Control; ICH, International Council for Harmonisation; Ileu, Isoleucine; IS, Internal Standard; KIC, α-ketoisocaproic acid; KIV, α-ketoisovaleric acid; KMV, α-keto-β-methylvaleric acid; LC-MS, Liquid chromatography-mass spectrometry; Leu, Leucine; Leu-d3, 5,5,5-trideuterio leucine; LLOQ, Lower Limit of Quantification; LQC, Lower Quality Control; MSUD, Maple syrup urine disease; MOI, Mode of ionization; MQC, Medium Quality Control; NBS, Newborn screening; QC, Quality Control; RBC, Red blood cells; SRM, Selected reaction monitoring; ULOQ, Upper Limit of Quantification; Val, Valine.

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Introduction

Maple syrup urine disease (MSUD) is a rare inherited metabolic disorder caused by defects in the metabolic pathways of branched-chain amino acids (BCAAs), leading to the accumulation of high levels of valine, leucine, and isoleucine in the blood. High levels of these amino acids over long periods lead to irreversible brain damage [1]. Classic MSUD progresses rapidly from birth, with newborns appearing asymptomatic initially but showing signs of metabolic intoxication, such as irritability and lethargy, within the first few days. Without treatment, this leads to severe encephalopathy, coma, and potentially fatal cerebral edema by the end of the first week [2]. Intermediate MSUD presents with less severe symptoms and may respond to dietary thiamine therapy, though affected individuals can still experience significant metabolic crises under stress.

The incidence of MSUD varies across different populations, with estimates ranging from 1 in 59,426 in Kuwait to 1 in 220,219 in the United States [3]. In South-West Germany, the incidence is approximately 1 in 119,573, while the nationwide incidence in Germany is 1 in 177,978. The prevalence of MSUD in the Czech Republic is reported to be 1 in 296,297 [4]. A study has reported that in India, 293 out of 2,105 examined patients were diagnosed with an inborn metabolic disorder, with 24 of those cases being MSUD [5]. This indicates that approximately 13.92 % of the patients examined had an inborn metabolic disorder, and 8.19 % of those with an inborn metabolic disorder had MSUD.

The alarming incidence of MSUD in India prompted us to undertake this investigation. The higher prevalence of MSUD in India compared to countries like the U.S. and Germany underscores the critical need for early detection and intervention in this population. Given the severe and often life-threatening complications associated with MSUD, including neurotoxicity and metabolic acidosis, it is imperative to focus on improving diagnostic methods in regions where the disease is more common.

BCAAs are essential amino acids that play a crucial role in various physiological and metabolic processes, including protein synthesis, muscle growth, and energy production [6]. BCAAs consist of three amino acids: leucine, isoleucine, and valine. These amino acids are primarily metabolized in skeletal muscle, where they serve as a readily available source of energy during exercise or in times of stress [7]. Branched-chain keto acids (BCKAs) are the corresponding ketogenic forms of BCAAs. BCKAs consist of α-ketoisocaproic acid (KIC, 4-methyl-2-oxovaleric acid), α-keto-β-methylvaleric acid (KMV, 3-methyl-2-oxovaleric acid), and α-ketoisovaleric acid (3-KIV, 3-methyl-2-oxobutanoic acid). They are produced during the breakdown of BCAAs and can be readily converted back to BCAAs through transamination (Fig. 1). BCKAs play a role in maintaining protein turnover and can be used as an alternative energy source, particularly in the brain [8].

MSUD is caused by mutations in the genes that code for the branched-chain alpha-keto acid dehydrogenase (BCKAD) complex, which is an enzyme complex responsible for breaking down BCAAs [9]. As a result of these mutations, the BCKAD complex is either absent or has reduced activity, leading to an accumulation of BCAAs and their corresponding alpha-keto acids in the blood and urine, resulting in the characteristic maple syrup odor that often accompanies MSUD [10]. The accumulation of BCAAs and BCKAs in MSUD can have a number of harmful consequences, including neurotoxicity, metabolic acidosis, and immune dysfunction [1,11,12]. Early detection and management of MSUD are crucial for ensuring the proper growth and well-being of affected individuals.

Newborn screening (NBS) plays a pivotal role in identifying MSUD, primarily through quantifying the ratios of (leucine + isoleucine) to alanine and phenylalanine concentrations on dried blood spots [2]. NBS is an essential public health measure that facilitates early identification and treatment of metabolic disorders like MSUD, significantly improving clinical outcomes. Dried blood spot (DBS) collection involves taking a small blood sample from a newborn's heel, which is then dried

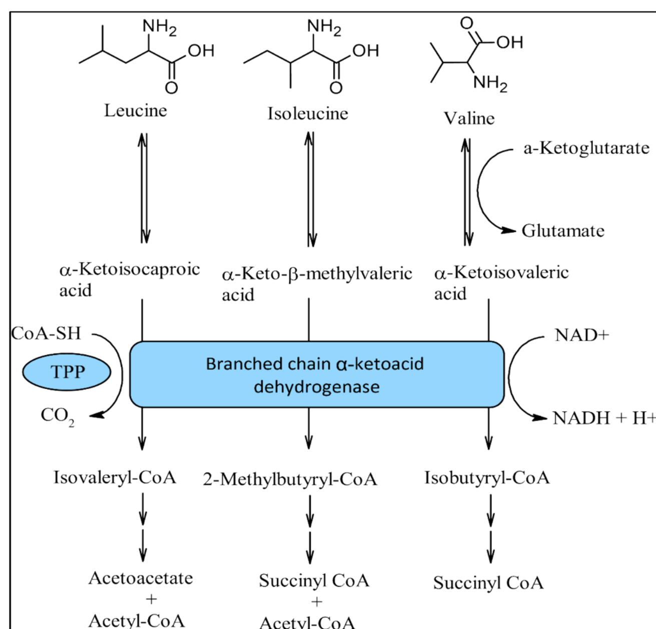

Fig. 1. Metabolism pathway of branched-chain amino acids: The branched-chain α-ketoacid dehydrogenase activity is decreased in maple syrup urine disease. Branched-chain amino acids (BCAAs) are transaminated to form corresponding branched-chain ketoacids (BCKAs) with the release of glutamate. The BCKAs are then further metabolized by the branched-chain α-ketoacid dehydrogenase (BCKAD) complex to form acetyl-CoA, succinyl-CoA, and acetoacetate, which can be used for energy production or further synthesis of other molecules.

on filter paper for analysis. This method is not only minimally invasive but also allows for easy storage and transport, making it ideal for widespread screening, especially in regions with limited healthcare infrastructure. However, due to the limitations of mass spectrometry in differentiating between leucine-isoleucine and hydroxyproline, neonates with isolated hydroxyprolinemia may trigger a false-positive NBS result for MSUD [13,14]. To confirm the diagnosis of MSUD and distinguish it from isolated hydroxyprolinemia, confirmatory amino acid analysis is essential. This analysis involves measuring the levels of BCAAs, alloisoleucine, and BCKAs in the blood. Abnormal levels of these metabolites strongly indicate the presence of MSUD [1,15–17].

In this work, we aimed to develop a liquid chromatography-mass spectrometry (LC-MS)-based analytical method to estimate the levels of BCAAs and BCKAs simultaneously from DBS samples. This method will be particularly valuable in the Indian context, where the high prevalence of MSUD necessitates efficient and reliable diagnostic tools to support early intervention efforts.

Materials and methods

Chemicals, reference standards, and reagents

Leucine (>95 %) (Leu), isoleucine (>95 %) (Ileu), valine (>95 %) (Val), α-ketoisovaleric acid (99 %) (KIV), α-keto-β-methylvaleric acid (99 %) (KMV), α-ketoisocaproic acid (99 %) (KIC) and formic acid 85 % (AR grade) were purchased from Sigma-Aldrich (St. Louis, MO, US). α-Keto isocaproic acid sodium salt (methyl-d3) (KIC-d3) (98 %) and (5,5,5-trideuterio leucine) (Leu-d3) were purchased from Cambridge Isotope Laboratories (Tewksbury, Massachusetts, US). LC-MS grade methanol was purchased from Biosolve BV, (Valkenswaard, The Netherlands). In-house Milli-Q water (Siemens Ultra Clear) was used. Luna PFP (150 mm × 4.6 mm, 3 μm) columns were purchased from Phenomenex (Torrance, California United States).

Instrumentation

A Thermo Scientific LTQ XL (Massachusetts, US) LC-MS with Dionex Ultimate 3000 liquid chromatograph interfaced with a linear ion trap analyser was used. The ionization source was heated electron spray ionization (HESI). MS/MS and chromatographic method development was performed using a Thermo Scientific XCalibur and Thermo Scientific Chromeleon software.

Preparation of standard solutions

Stock solutions (5 mg/mL) of BCAAs (Leu, Ileu, Val), BCKAs (KIV, KIC and KMV) and their internal standards (KIC-d3, Leu-d3) were prepared in methanol. Working stocks of concentration 1 mg/mL were prepared in 50:50 v/v methanol: water and stored at − 20℃ until use. Calibration solutions were prepared for BCAAs and BCKAs in the range of 2 to 500 μM and 1 to 250 μM, respectively. Quality control samples such as Low-Quality Control (LQC), Medium-Quality Control (MQC), and High-Quality Control (HQC) were prepared at 50, 250, and 450 μM for BCAAs and 35, 150, 220 μM for BCKAs. Calibration and quality control (QC) working solutions were mixed with leftover blood from healthy controls (hematocrit of 50 %) at a ratio of 1:40 (% v/v). This 1:40 (v/v) ratio was selected to optimize analyte concentrations within the detection range of the analytical method, minimize matrix effects, prevent overloading of the analytical system, and ensure consistency with routine sample processing protocols [18]. DBS calibrators and QCs were prepared by pipetting 40 μL of the blood mixtures onto Whatman 903 filter paper. Prior to the analysis, DBS cards were left to dry at room temperature for 3 h. Plasma was prepared by centrifuging the human whole blood collected in K2 EDTA tubes. Calibration standards and quality control samples were prepared by spiking pooled plasma with the respective working standards of BCAAs and BCKAs. Internal standard (IS) solutions were prepared at a concentration of 50 μM in 50:50 v/v methanol: water.

Sample collection and processing

The institutional ethics committee of Kasturba Medical College, Manipal, granted approval for this study (MUEC/010/2017 dated 08.05.2017, MAHE EC/Renewal-02/2018 dated 10.05.2018, and IEC 303/2019 dated 15.05.2019). Blood sampling from neonates was done by certified medical professionals 48 h after birth in Kasturba hospital, Manipal, India. Informed consent from parents was obtained before sampling. Blood was collected in EDTA-coated vacutainers. For the preparation of DBS, 40 μL of the blood was spotted onto Whatman 903 filter paper. The DBS samples were dried and stored at − 80℃ until further analysis. The remaining blood was converted to plasma by centrifuging at 7000 × *g* at 4 °C and stored at − 80℃ until further analysis.

Plasma samples that had been frozen at −80℃ were thawed to room temperature, and vortexed for 2 min. 20 μL of the IS solution were added to 180 μL plasma sample and vortexed for 2 min. In order to denature the protein, methanol was added to the sample in 1:3 ratio followed by vortexing for 2 min. The final content was centrifuged at 9392 × g, at 4℃ for 10 min and 20 μL of supernatant was injected into LC-MS system.

For processing DBS samples, two DBS discs of 3.2 mm (2 × 3.2 mm) were punched into a 96-well plate (1.2 mL volume). The analytes were extracted by adding 200 μL of a working standard solution of IS prepared in methanol: 0.1 % formic acid buffer (75:25 v/v) and shaking it for 2 h at 1000 RPM. Various solvent combinations were initially tested to optimize extraction efficiency before selecting methanol and formic acid as the final extraction solution. The choice was based on achieving the highest recovery rates and consistency across multiple trials. The preliminary workup data, detailing the different combinations tested and their respective % recovery results, is provided in Supplementary Table S1. After shaking, the contents of the 96-well plate were sonicated for 2 min at 25℃ and then transferred to a 0.5 mL microcentrifuge tube. The tube was centrifuged at 9392 × *g* at 4℃ for 10 min, and 20 μL of supernatant was injected into the LC-MS system.

Chromatographic and mass spectrometric conditions

Knowledge on the physicochemical properties of analytes plays a crucial role in selecting a suitable column and mobile phase. The LogP vale and pKa values are of paramount importance. Leucine (Molecular weight 131.17 g/mol) has three hydrogen bond acceptors and two hydrogen bond donors. It has a logP value of −1.52 and pKa value of 2.79. Isoleucine (Molecular weight 131.17 g/mol) has three hydrogen bond acceptors and two hydrogen bond donors. It has a logP value of −1.70 and pKa value of 2.79. Valine (Molecular weight 117.14 g/mol) has three hydrogen bond acceptors and two hydrogen bond donor. It has a logP value of −2.26 and pKa value of 2.72. KIV (Molecular weight 116.11 g/mol) has three hydrogen bond acceptors and one hydrogen bond donor. It has a logP value of 1.31 and pKa value of 3.37. KMV (Molecular weight 130.14 g/mol) has three hydrogen bond acceptors and one hydrogen bond donor. It has a logP value of one and pKa value of 3.52. KIC (Molecular weight 130.14 g/mol) has three hydrogen bond acceptors and one hydrogen bond donor. It has a logP value of 0.82 and pKa value of 3.53. A summary of physicochemical properties of the BCAAs and BCKAs delineated above is shown in Supplementary Table S2.

Chromatographic separation was performed using a Luna PFP (150 mm × 4.6 mm, 3 μm) column. A linear gradient of mobile phase A (methanol), mobile phase B (0.1 % formic acid in water adjusted to pH 2.76) and mobile phase C (water) was applied (Table 1). The column and autosampler temperature were maintained at 40 °C and 4 °C, respectively. The total run time was 10 min.

The mass spectrometer was operated in positive/negative switching mode with selected reaction monitoring (SRM). The electrospray ionization (ESI) used a spray and capillary voltage of 3.5 V and 14 V, respectively; vaporizer and ion transfer capillary temperature of 310 °C and 350 °C, respectively; nitrogen sheath gas, auxiliary gas and sweep gas flow of 65, 11 and 2 arbitrary units, respectively; tube lens and multipole 00 offset voltage of 80.00 V and −13.20 V, respectively. The SRM transitions, along with the respective mode of ionization (MOI) and collision energy (CE) used for the biomarkers and the internal standards, are shown in Table 2.

Method validation

The developed LC-MS analytical method was validated for accuracy, precision, linearity, and recovery, as per the recommendations of ICH M10 Bioanalytical method validation guideline. Linearity, recovery, and accuracy were assessed using the blank subtraction technique, where endogenous analyte levels in healthy control blood samples were determined via the standard addition method. This enabled subtraction of the endogenous analyte to IS ratio from the spiked DBS analyte to IS ratio. Regression analysis was then conducted on the resultant subtracted area ratio versus analyte concentration plot. The percentage nominal for each level was calculated. Linearity experiments were carried out in five batches, each comprising blank, zero, and ten nonzero

Table 1
Gradient elution program for the chromatographic separation of BCAAs and BCKAs.

Time (min)	Flow (ml/min)	%A	%B	%C
0	0.2	75	25	0
5.5	0.2	75	25	0
6.2	0.1	90	0	10
9	0.2	10	0	90
10	0.2	75	25	0

Table 2
Mass transition, collision energy, ionization mode, internal standard, and retention time for BCAAs, BCKAs, and their internal standards.

Analyte	SRM transitions	Collision energy	Mode of ionization	Internal standard	Retention time (mins)
Leu	131.68 → 84.9	20	+	Leu-d3	7.59
Ile	131.68 → 84.9	20	+	Leu-d3	6.83
Val	117.89 → 71.83	20	+	Leu-d3	4.48
KMV	129 → 129	25	−	KIC-d3	3.28
KIC	129 → 84	35	−	KIC-d3	5.13
KIV	115.86 → 87	35	−	KIC-d3	2.23
Leu-d3	136 → 90	20	+		7.53
KIC-d3	155 → 130	35	−		2.87

samples. Accuracy evaluations were performed at different QC levels such as Lower Limit of Quantification (LLOQ), LQC, MQC, and HQC by comparing mean calculated values (post-endogenous subtraction) to nominal concentrations. Extraction recovery and matrix effect were assessed at varying QC levels using comparisons with neat standard solutions and post-extraction spike techniques, respectively. These assessments were undertaken to ensure the method's robustness and reliability [18,19].

Evaluation of dilution integrity

Dilution integrity was assessed to verify the ability of analyte concentrations exceeding the Upper Limit of Quantification (ULOQ) in actual samples to be accurately back-calculated to their original levels. The dilution integrity QCs were prepared in plasma and DBS (n = 5) at concentrations of 1,000 μM and 500 μM, respectively, for BCAAs and BCKAs. Following the extraction of the analytes from the samples, serial dilution was performed at a ratio of 1:5 on the final extracts by adding an appropriate volume of plasma [20]. Subsequently, the samples underwent processing and analysis as previously described, and the concentrations of the dilution QCs were back-calculated. As per ICH M10 guidelines, the precision and accuracy of the back-calculated dilution QCs should not deviate beyond ± 15 %.

Assessment of relative matrix effect

To investigate the influence of various components within plasma and DBS matrices on the mass spectrometric ionization responses of BCAAs and BCKAs, we spiked all the analytes at calibration ranges (BCAAs: 2–500 μM and BCKAs: 1–250 μM) into blood and plasma obtained from five different healthy neonates. Each concentration point per blood sample was analyzed once by LC-MS, resulting in a total sample size (n) of 5 at each concentration level. Standard curves were constructed for each blood sample by fitting the obtained data to a straight line using $1/x^2$ weighted least squares. To evaluate relative matrix effects, the standard lines were generated using five different lots of biofluid. For the assay to be considered free of relative matrix effects, the slopes of the standard lines obtained for each lot of biofluid should not exceed 3–4 % (relative standard deviation), and each individual concentration point should vary by less than 15 % across all five lots of biofluid, except at the lower limit of quantitation where 20 % is acceptable [21].

Hematocrit effect (HCT)

According to gestational age and clinical settings, the HCT in newborns varies from 28 to 65 % within the first 28 days after birth [22]. Hematocrit (HCT) is defined as the percentage of red blood cells (RBCs) in the total volume of blood. The distribution of blood on the DBS card is significantly influenced by the HCT in DBS-based analysis, which introduces assay bias [23,24]. Hence, extra care is taken while analyzing DBS samples to reduce bias. The viscosity of whole blood is influenced by its HCT concentration, which in turn affects the size of the DBS spot and thus the concentration of the analyte. To prepare various hematocrit levels, red blood cells (RBCs) and plasma were separated through centrifugation, and the hematocrit (RBC ratio) was measured using a microhematocrit reader [25]. Appropriate volumes of plasma were then added or removed to achieve the desired ratios. The process was repeated, adjusting volumes accordingly, to obtain hematocrit levels ranging from 30 % to 65 % [26]. The impact of altering HCT levels on the test was examined at eight HCT levels (30, 35, 40, 45, 50, 55, 60, and 65 % v/v), with concentrations at LQC and HQC, checking for consistency in analytical results.

Effect of blood volume and punch position

To assess the effect of blood volume on spot size and analytical results, 10–60 μL of whole blood samples were applied to DBS cards in five replicates at low and medium quality control levels [24]. Smaller volumes (10 μL) produced smaller spots, primarily serving to study the influence of spot size on analysis rather than for routine testing. The impact of punch position was evaluated using 40 μL DBS samples (n = 5) at low and high quality control levels. This specific volume was selected to ensure that the blood spot was sufficiently large to allow for consistent and reliable punches from multiple locations on the DBS card. To assess the punch position effect, punches were taken from the center of the spot as well as from two distinct peripheral positions (Peripheral 1 and Peripheral 2). The results from these different punch locations were compared to determine if the position of the punch within the blood spot influenced the accuracy and precision of the analytical measurements. This analysis was crucial in verifying the robustness of the method, ensuring that variations in punch location do not significantly affect the outcomes. The DBS sample collection criteria were adhered to during the clinical sample collection and processing. Low-quality samples, such as circles without blood, filter paper without saturation, filter paper damage, stacked specimens, contamination, serum rings, and clotted specimens were rejected [27,28].

Stability studies

Benchtop stability was evaluated on LQC and HQC samples stored at room temperature (25 °C) for 2, 4, and 8 h. Stability of stock solution on storage at (2–8 °C) was evaluated on six replicates of freshly prepared MQC samples for 2, 4, 6, and 8 h. Processed sample stability in the autosampler was evaluated at 4 °C for 6, 12, 24, and 48 h on LQC and HQC samples. For the freeze–thaw stability study, LQC and HQC samples were subjected to three freeze–thaw cycles at −80 °C. After each freeze–thaw cycle, the samples were thawed unassisted at room temperature and analyzed. The long-term stability of analytes in DBS and plasma at −80 °C was studied for a duration of up to 360 days to assess the reliability of DBS samples for biobanking and retrospective analyses. LQC and HQC samples were stored at −80 °C and analyzed at 30, 60, 90, 180, and 360 days. On the day of analysis, samples were thawed unassisted at room temperature and analyzed. The mean, standard deviation, and percent change were calculated for each study to evaluate stability.

Evaluation of interconvertibility between the plasma & DBS assay

To evaluate the interconvertibility of the results obtained from DBS and plasma, we compared the BCAA and BCKA concentrations measured in DBS specimens with those of plasma. Residual blood from 15 anonymous adults was collected in K2 EDTA tubes from the Department of Biochemistry, Kasturba Medical College, Manipal. A DBS card was prepared by spotting 40 μL of blood onto the 903 paper, and the rest was centrifuged to obtain plasma. The plasma and DBS samples were analyzed, and the concentrations of BCAAs and BCKAs in both matrices were calculated. Pearson correlation coefficient and linear regression equations were used to predict the concentration in plasma corresponding to the concentration in the DBS. Further, Bland-Altman

comparison was made between the concentrations determined from both matrices to evaluate the distribution of measurements from the mean at a 95 % confidence interval.

Determination of reference range for BCAAs and BCKAs

Using the validated method, we analyzed 350 DBS samples to build a reference range for analytes in the healthy newborn population of Udupi, a coastal district in South India. We included healthy term neonates (3–10 days old) on breastfeeding, born between 37 and 42 weeks of gestation, with a birth weight greater than 2,500 g, with or without physiological jaundice, and born by both cesarean and vaginal delivery. We excluded neonates with asphyxia, those requiring IV fluids, and neonates with congenital malformations. The reference intervals for the metabolites in DBS, calculated using the nonparametric percentile method with a 95 % confidence interval, are reported in terms of the 2.5th to 97.5th percentiles.

Greenness of analytical procedure

The environmental impact of the proposed analytical method was evaluated using the AGREE software (Analytical GREEnness Metric Approach and Software) [29]. This method considers twelve criteria, each scored from zero to one, with higher scores indicating greater alignment with ecological principles. The evaluation assessed the use of toxic substances, waste generation, and resource consumption against predefined criteria. Penalty points were assigned based on adherence, culminating in a final score displayed at the centre of a circular pictogram. In this pictogram, criteria where the method aligns with green chemistry principles are coloured green, deviations are marked red, and intermediate scores are shown with gradations in between, providing a clear and nuanced assessment.

Results and discussion

LC-MS/MS method

As observed from the physicochemical properties of BCAAs and BCKAs (Supplementary Table S2), all six analytes are polar with slight lipophilic properties. Accordingly, a reversed phase Luna PFP column was selected as the stationary phase. The PFP column has a pentafluoro phenyl group bonded to a trimethyl silane skeleton and provides sufficient selectivity for BCAAs and BCKAs.

A linear mobile phase gradient was employed to achieve efficient separation within a short run time. The initial mobile phase composition started with a higher proportion of mobile phase A (methanol) and a lower proportion of mobile phase B (0.1 % formic acid in water adjusted to pH 2.76). This composition aids in the initial elution and separation of the analytes. The 0.1 % formic acid in mobile phase B serves two purposes: it slightly reduces the pH of the mobile phase, which can influence the ionization state of the analytes and impact their retention on the column; and it acts as an ion-pairing agent, further enhancing the separation of analytes. The specific gradient profile is optimized to ensure adequate resolution and separation of the analytes of interest within the given run time.

SRM (Selective Reaction Monitoring) analysis, which is a highly sensitive and selective MS technique, was adopted for the quantification of the biomarkers. The SRM transitions and the respective CE used for the biomarkers and the internal standards, along with their retention times, are detailed in Table 2. Chromatograms of all the six analytes along with their respective internal standards is provided in Fig. 2.

Method validation

Accuracy and precision

Six replicates of QC samples were injected at LLOQ, LQC, MQC, and HQC to assess intra-day accuracy and precision. Three separate runs for accuracy and precision over successive days were carried out to establish inter-day accuracy and precision. An acceptable limit of $\pm$ 15 % accuracy ($\pm$20 % for LLOQ) and $\leq$ 15 % Coefficient of Variation (%CV) precision was used to evaluate the bias of calculated concentrations with the nominal concentrations of QCs. Prior to each accuracy and precision run, a linearity run was performed to ensure concentrations fell within the established linear range. The intra-day precision (%CV) in plasma was found to be less than 5.98 %, with accuracy ranging from 90.21 % to 109.38 %. Inter-day precision (%CV) was less than 5.84 %, with accuracy ranging from 90.32 % to 109.14 % for all six analytes. In DBS, the intra-day precision (%CV) was less than 5.71 %, with accuracy ranging from 90.57 % to 108.78 %, while inter-day precision (%CV) was less than 5.73 %, with accuracy ranging from 92.67 % to 108.41 % for all six analytes. The results, presented in Supplementary Table S4, indicate that both the repeatability and reproducibility of this method for the quantitation of BCAAs and BCKAs from plasma and DBS are satisfactory.

Linearity and LLOQ

Calibrators for BCAAs and BCKAs were prepared within the range of 2–500 μM and 1–250 μM concentrations, respectively. Three independent calibration curves (CCs) were plotted on three separate days. Using XCalibur software and applying a weighting factor of $1/x^2$, the slope and intercept of the linearity curves were determined. The calibration range, linearity equations, and correlation coefficients (R values) for all six analytes in both DBS and plasma are provided in the Supplementary Table S3. The selected calibration ranges for the analytes cover the published reference ranges: 45–214 μM for Leu, 13–135 μM for Ileu, 61–235 μM for Val, 6.5–12.3 μM for KIV, 16.3–28.7 μM for KIC and 8.7–16.7 μM for KMV [30,31]. The correlation coefficients obtained were within the specified limits (R > 0.99) demonstrating linearity of the method. The back-calculated concentrations of calibrator standards were within 20 % of the nominal concentration for LLOQ and within 15 % for the other CC standards, confirming the suitability of the technique within the tested range.

Recovery

A recovery study was conducted to assess the degree and uniformity of analyte and IS extraction from the matrix. The extraction efficiency of analytes was determined by analysing five replicates of plasma and DBS samples at three concentration levels (low, medium and high QC). This determination was made by calculating the ratio between the response of analytes (adjusted for endogenous level) in samples spiked before extraction and those spiked after extraction [32]. In plasma samples, the mean recovery percentages were found to be 97.46 %, 96.99 %, 98.35 %, 97.36 %, 92.86 %, and 99.32 % for Leu, Ileu, Val, KIV, KMV, and KIC, respectively. Meanwhile, in DBS samples, the mean recovery percentages were found to be 97.44 %, 91.19 %, 92.49 %, 94.05 %, 90.56 %, and 95.89 % for the same analytes. These results suggest that the chosen extraction method efficiently retrieves the target analytes, with high recovery (>90 %) observed in both plasma and DBS samples [33]. The slightly lower recoveries in DBS compared to plasma are likely due to the dried blood matrix presenting additional challenges during extraction. Despite this, the %CV values were within acceptable limits (<20 %), indicating consistent and reproducible recovery across different concentration levels [34]. This reproducibility is crucial for ensuring the reliability and accuracy of the analytical method across multiple samples and concentrations. The breakdown of plasma and DBS recovery percentages across various QC levels is detailed in Supplementary Table S4, providing a comprehensive view of the extraction efficiency at different concentration levels. Overall, the results demonstrate the method's reliability in quantifying the analytes of interest in both plasma and DBS samples.

Dilution integrity

To ensure the validity of analysis on diluted samples, the impact of

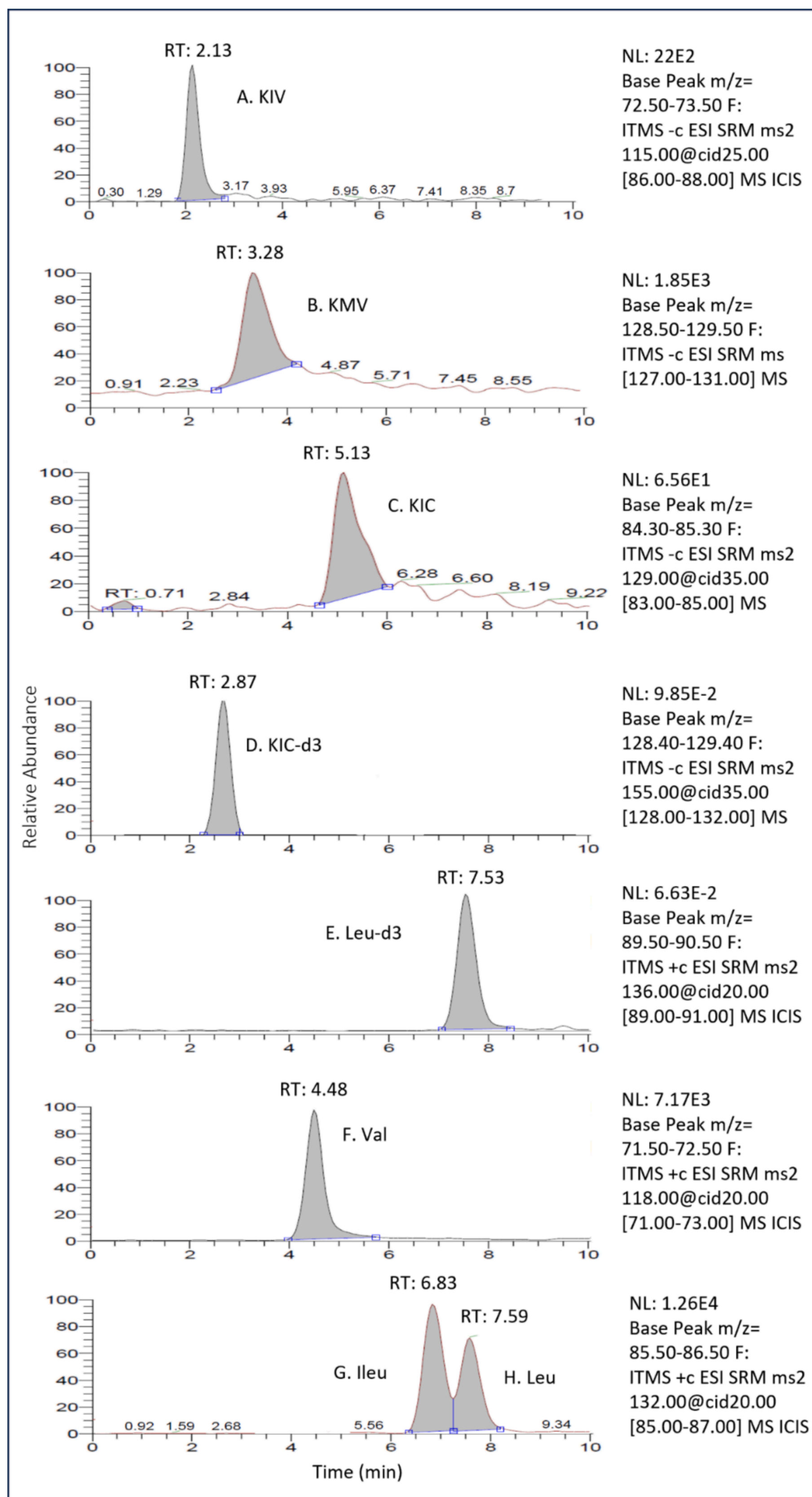

Fig. 2. Chromatograms at LLOQ for (A) KIV (RT 2.13 min), (B) KMV (RT 3.28 min), (C) KIC (RT 5.13 min), (D) KIC-d3 (RT 2.87 min), (E) Leu-d3 (RT 7.53 min), (F) Val (RT 4.48 min), (G) Ileu (RT 6.83 min), (H) Leu (RT 7.59 min).

the dilution process on both accuracy and precision was meticulously evaluated. This assessment revealed that the dilution procedure exerted no statistically significant influence on the accuracy or precision of the analysis, irrespective of the sample matrix (plasma or DBS). The average calculated concentrations of BCAAs and BCKAs closely aligned with anticipated ranges, signifying the effectiveness of dilution in bringing spiked samples within the measurable limits without jeopardizing the fidelity of the results. Additionally, the determination's accuracy and precision remained within acceptable parameters. This substantiates the effectiveness of the dilution process in adjusting spiked samples to a measurable range while preserving data integrity. Table 3 summarizes the mean measured concentration, accuracy, and precision for the samples undergoing dilution integrity assessment.

Matrix effect

In our validation analysis, we observed that the slopes of the standard curves derived from the five distinct blood samples exhibited a variance of less than 3.64 %, as outlined in Supplementary Table S5. This pattern implies a stable response across various batches of biofluid, suggesting the absence of significant matrix effects. Furthermore, the precision of individual concentration measurements remained within the acceptable margin of 15 %, as depicted in Supplementary Table S6. This reinforces the reliability of our methodology and its capability to accurately measure analyte concentrations across diverse biofluid batches. These findings, aligned with widely accepted standards, affirm that our approach is devoid of relative matrix effects. Thus, any observed disparities in ionization responses are likely due to the inherent nature of the analytes rather than differences in the blood and plasma matrices. Consequently, our method offers a dependable means to precisely quantify BCAAs and BCKAs concentrations in both plasma and DBS matrices, thereby expanding its utility in clinical and research domains.

Effect of HCT, blood volume and punch position

The impact of HCT levels, ranging from 30 % to 65 % v/v, on the analysis outcomes assessed at LQC and HQC concentrations, has been scrutinized. It was found that the back-calculated results remained within the accuracy level of 15 % and demonstrated a CV not exceeding 15 %. This comprehensive evaluation conclusively illustrates that variations in HCT levels do not compromise the accuracy of the analysis for BCAAs and BCKAs. Such findings are particularly noteworthy given the method's potential application in testing neonates whose physiological parameters are dynamically evolving. The graphical representation of these results is presented in Fig. 3. Furthermore, the investigation into the impact of blood volume and punch position, depicted in Figs. 4 and 5, respectively, revealed differences from nominal concentrations within ± 3 %. This indicates that neither blood volume nor punch position significantly influences the analysis outcomes, further consolidating the method's reliability and suitability for diverse testing scenarios. The DBS sample collection criteria were adhered to during the clinical sample collection and processing. Low-quality samples like circles without blood, filter paper without saturation, filter paper damage, stacked specimens, contamination, serum rings, and clotted specimens were rejected [25,26].

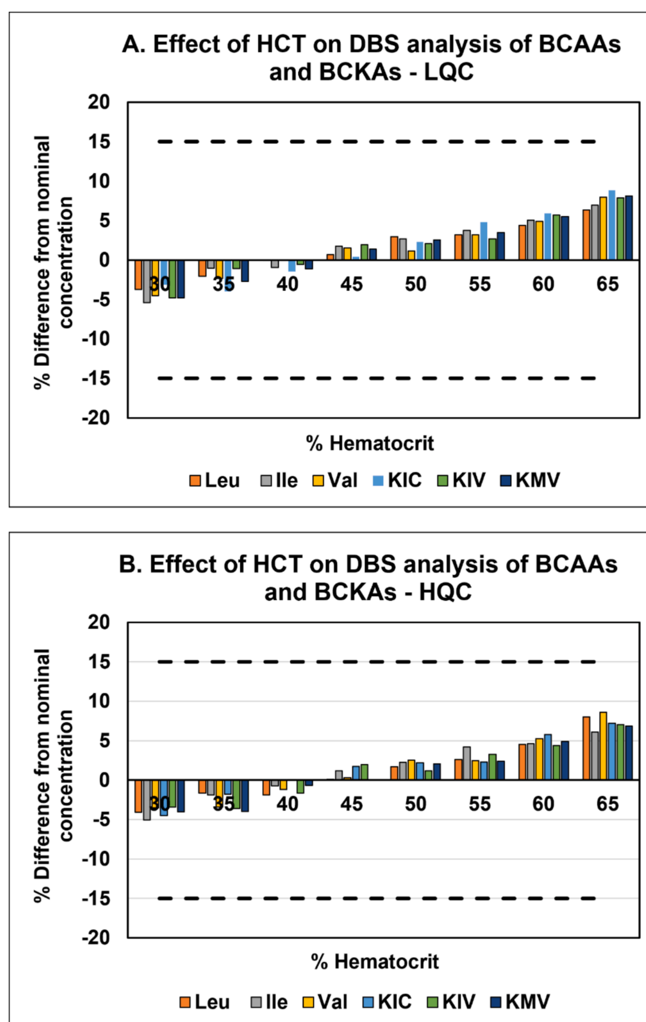

Fig. 3. Histograms representing the effect of hematocrit on the DBS (n = 5) assay results of BCAAs and BCKAs at (A) LQC and (B)HQC levels.

Stability

A robust evaluation of biomarker stability is paramount, encompassing both analytical conditions and storage parameters. To this end, we conducted comprehensive stability studies adhering to recommended FDA stress conditions. The analytes demonstrated remarkable stability in methanol stock solutions, with a percent change of less than 5 % from the baseline within eight hours, as illustrated in Supplementary Figure S1. This implies a high degree of resilience against potential degradation during sample preparation.

Benchtop stability studies revealed a remarkably consistent profile for both DBS and plasma matrices (Supplementary Figures S2, S3). With a marginal decrease of less than 5.5 % observed over the eight-hour test, these findings underscore the suitability of the biomarkers for routine

Table 3
Mean calculated concentration, accuracy, and precision for the dilution integrity samples of BCAAs and BCKAs.

Analyte	Spiked Conc.	Plasma			DBS		
		Mean calculated Conc. (µM)	Accuracy (%)	Precision (%CV)	Mean calculated Conc.	Accuracy (%)	Precision (%CV)
Leu	1000	924.1068	92.4 ± 1.5	1.53	1066.2634	106.6 ± 6.3	3.71
Ileu	1000	1025.6761	102.5 ± 6.7	1.67	919.9704	91.9 ± 4.9	1.15
Val	1000	973.7066	97.3 ± 4.6	5.52	991.3817	99.1 ± 6.8	2.78
KIV	500	478.9856	95.7 ± 5.1	6.83	508.7793	101.7 ± 5.2	3.15
KMV	500	476.0073	95.2 ± 4.6	4.29	477.374	95.4 ± 4.8	1.28
KIC	500	492.003	98.4 ± 4.6	5.52	459.4898	91.8 ± 7.6	0.72

Note: Leucine (Leu), isoleucine (Ileu), valine (Val), α-ketoisovaleric acid (KIV), α-keto-β-methylvaleric acid (KMV), α-ketoisocaproic acid (KIC).

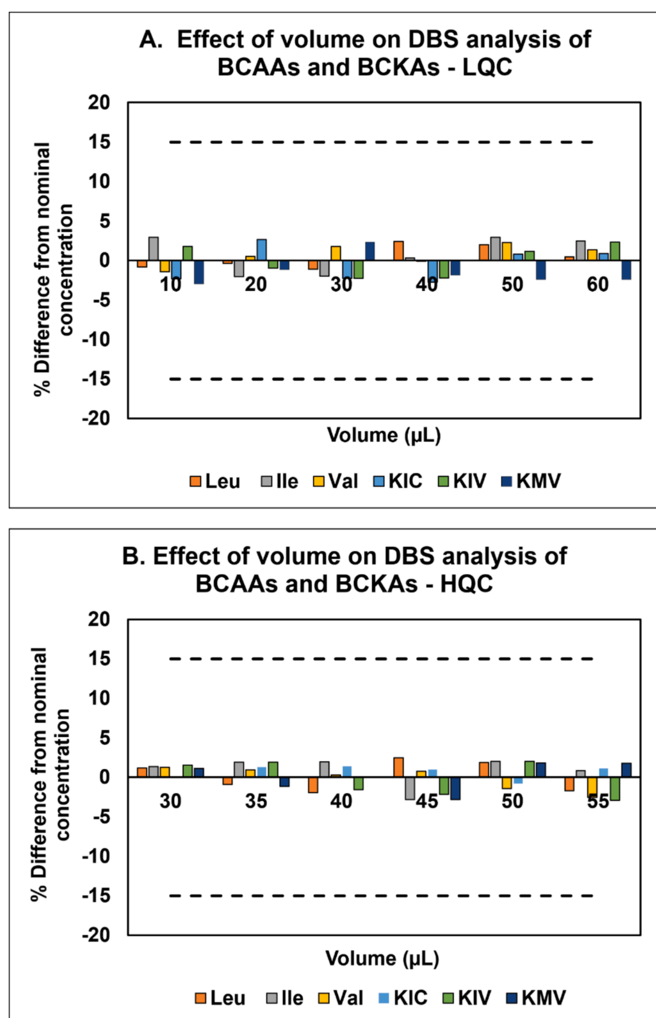

Fig. 4. Histograms representing the effect of blood volume on the DBS (n = 5) assay results of BCAAs and BCKAs at (A) LQC and (B) HQC levels.

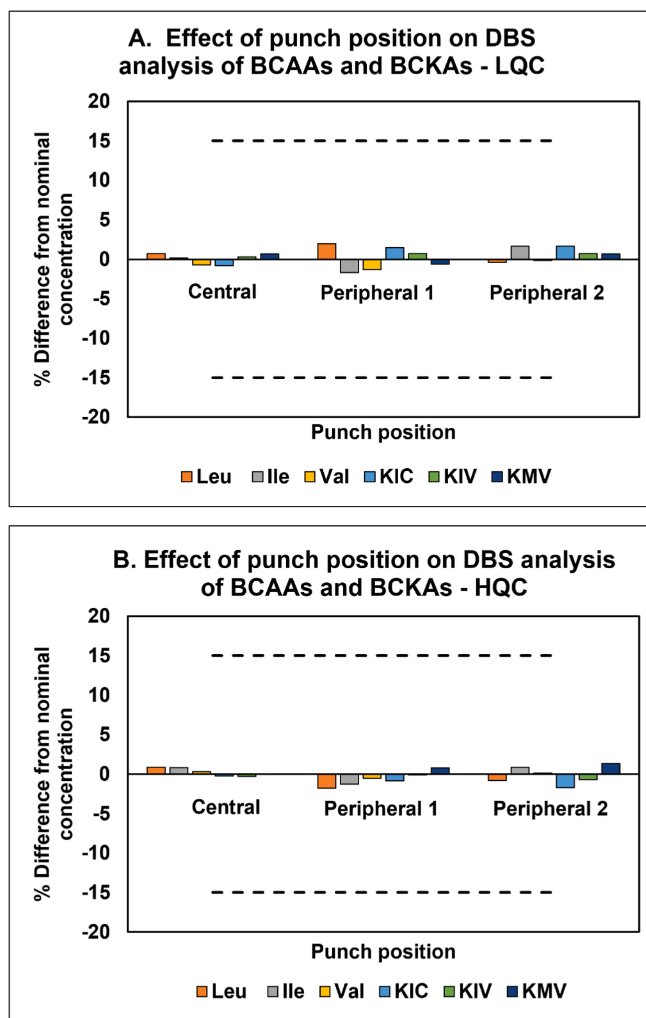

Fig. 5. Histograms representing the effect of punch position on the DBS (n = 5) assay results of BCAAs and BCKAs at (A) LQC and (B) HQC levels.

laboratory workflows, minimizing potential analyte loss during processing.

The impact of autosampler residence time at 4 °C was evaluated, showing that all biomarkers remained stable within 5 % at both LQC and HQC levels for up to 48 h, enhancing compatibility with automated analytical systems (Supplementary Figures S2, S3).

Long-term stability on storage at −80 °C for a period of 360 days produced exceptional results (Supplementary Figures S2, S3) for both DBS and plasma samples. The change was within 8 % and 14 % for DBS and plasma samples respectively, at both quality control levels, with DBS demonstrating superior stability on long-term storage. DBS offers additional advantages, including easier sample collection and reduced need for cold chain transportation, which is particularly beneficial in resource-limited settings.

The freeze–thaw stability study further exemplified the robustness of the analytes. All biomarkers exhibited changes of less than 12 % (Supplementary Table S7) after three freeze–thaw cycles from −80 °C to room temperature. This translates to minimal analyte degradation without a compromise in data integrity under potentially extreme sample handling conditions.

Overall, the stability profiles significantly exceed the accepted threshold of ± 15 %, demonstrating exceptional stability of the biomarkers in both DBS and plasma. However, DBS stands out for its superior long-term stability and logistical advantages, making it a compelling choice for biomarker analysis, especially in settings with logistical constraints.

Evaluation of interconvertibility between the plasma & DBS assay

Our investigation employed the Pearson correlation coefficient to assess the relationship between DBS and plasma analyte concentration. Subsequently, we generated linear regression equations for each analyte (Fig. 6). The resulting equations and their corresponding R^2 values offer critical insights into the accuracy and dependability of translating DBS concentrations into their corresponding plasma values. The regression equations and R^2 values are as follows: (Leu: $y = 0.9966x + 0.0848$, $R^2 = 0.9943$; Ileu: $y = 0.9968x + 0.3085$, $R^2 = 0.9950$; Val: $y = 0.9888x + 0.1825$, $R^2 = 0.9991$; KIC: $y = 1.0132x - 1.1175$, $R^2 = 0.9706$; KIV: $y = 0.8593x + 2.3702$, $R^2 = 0.8929$; KMV: $y = 1.0862x - 1.9940$, $R^2 = 0.9116$). Here, y is the plasma concentration and x is the DBS concentration. The remarkably strong correlations observed between the concentrations of BCAAs and BCKAs in both DBS and plasma matrices signify a high degree of comparability. This indicates the viability of using DBS as a reliable method for estimating plasma concentrations of these analytes.

To ensure the robustness of the regression analysis, outliers were checked using standardized residuals, considering values greater than ± 3 as potential outliers [35], and found no points that deviated significantly from the regression line. This confirms the reliability of the correlation between DBS and plasma concentrations.

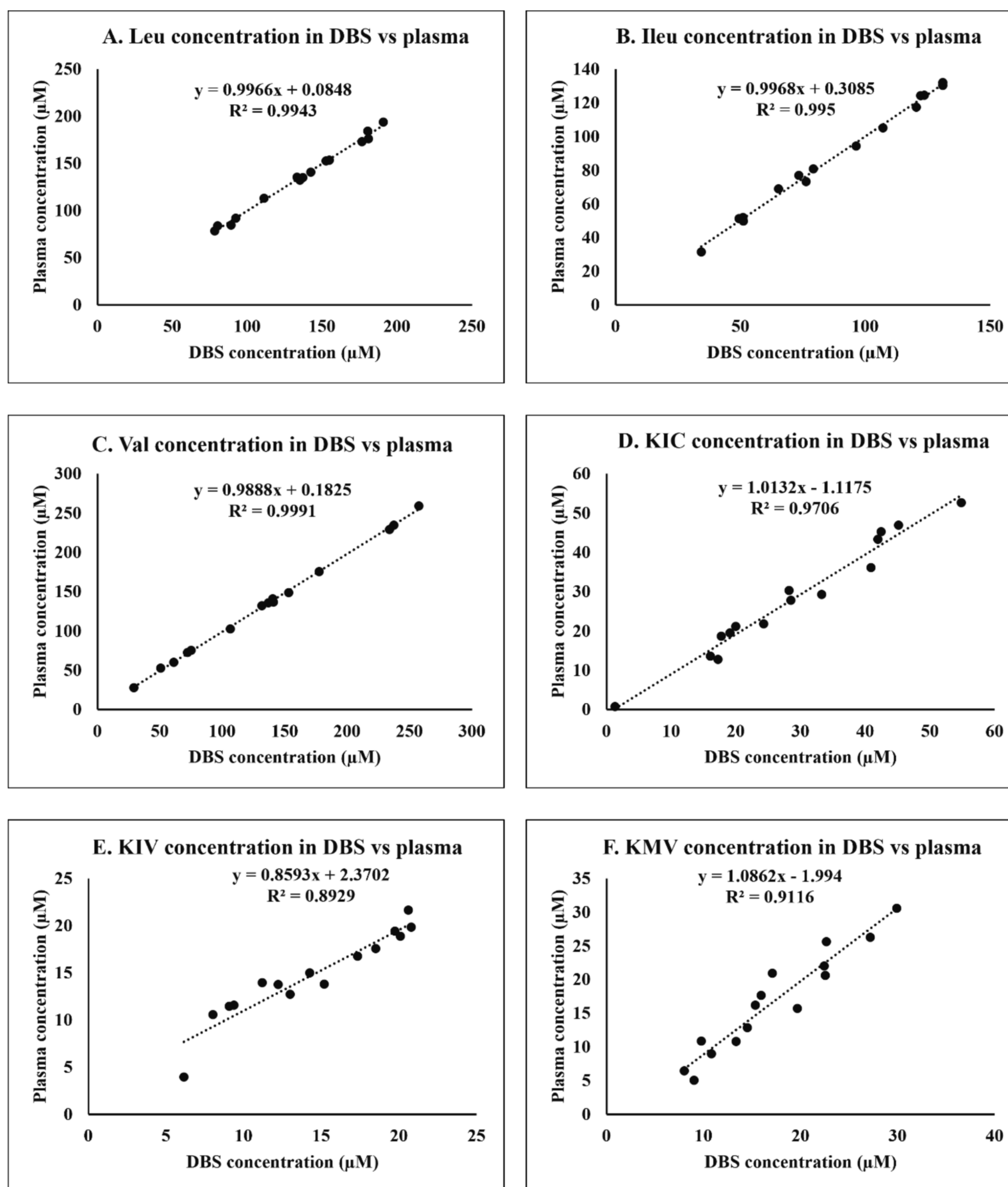

Fig. 6. Linear regression of concentration of (A) Leucine, (B) Isoleucine, (C) Valine, (D) KIC, (E) KIV and (F) KMV in DBS versus plasma.

To further substantiate the concordance between DBS and plasma methodologies, we conducted a Bland-Altman analysis. This analysis yielded mean ± SD values for BCAAs and BCKAs in DBS compared to their corresponding values in plasma, as detailed below:

Leucine: 135.5957 ± 38.2300 µM (DBS) vs. 135.2131 ± 38.2300 µM (plasma).

Isoleucine: 87.4391 ± 33.4387 µM (DBS) vs. 87.4661 ± 33.4145 µM (plasma).

Valine: 133.5634 ± 70.5288 µM (DBS) vs. 132.2505 ± 69.7718 µM (plasma).

KIC: 28.7258 ± 14.2371 µM (DBS) vs. 27.9878 ± 14.6417 µM (plasma).

KIV: 14.3694 ± 4.9679 µM (DBS) vs. 14.7183 ± 4.5177 µM (plasma).

KMV: 17.2123 ± 6.6932 µM (DBS) vs. 16.7018 ± 7.6143 µM (plasma).

The analysis revealed a mean bias of 0.3826, −0.0270, 1.3129, 0.7381, −0.3489, and 0.5105 for leucine, isoleucine, valine, KIC, KIV, and KMV, respectively. These findings from the Bland-Altman analysis demonstrate an acceptable level of agreement between DBS and plasma methodologies for measuring all six analytes. The limit of agreement (LOA) fall within range of ± 15. The minimal mean bias observed for each analyte signifies an absence of any systematic discrepancy between the two methods. Furthermore, the LOA computed as the mean bias ± 1.96 SD, were found to be relatively narrow demonstrating the precision between the two methods. Visual representations of the Bland-Altman analysis for each analyte are provided in Fig. 7.

In essence, the collective weight of our evidence strongly suggests

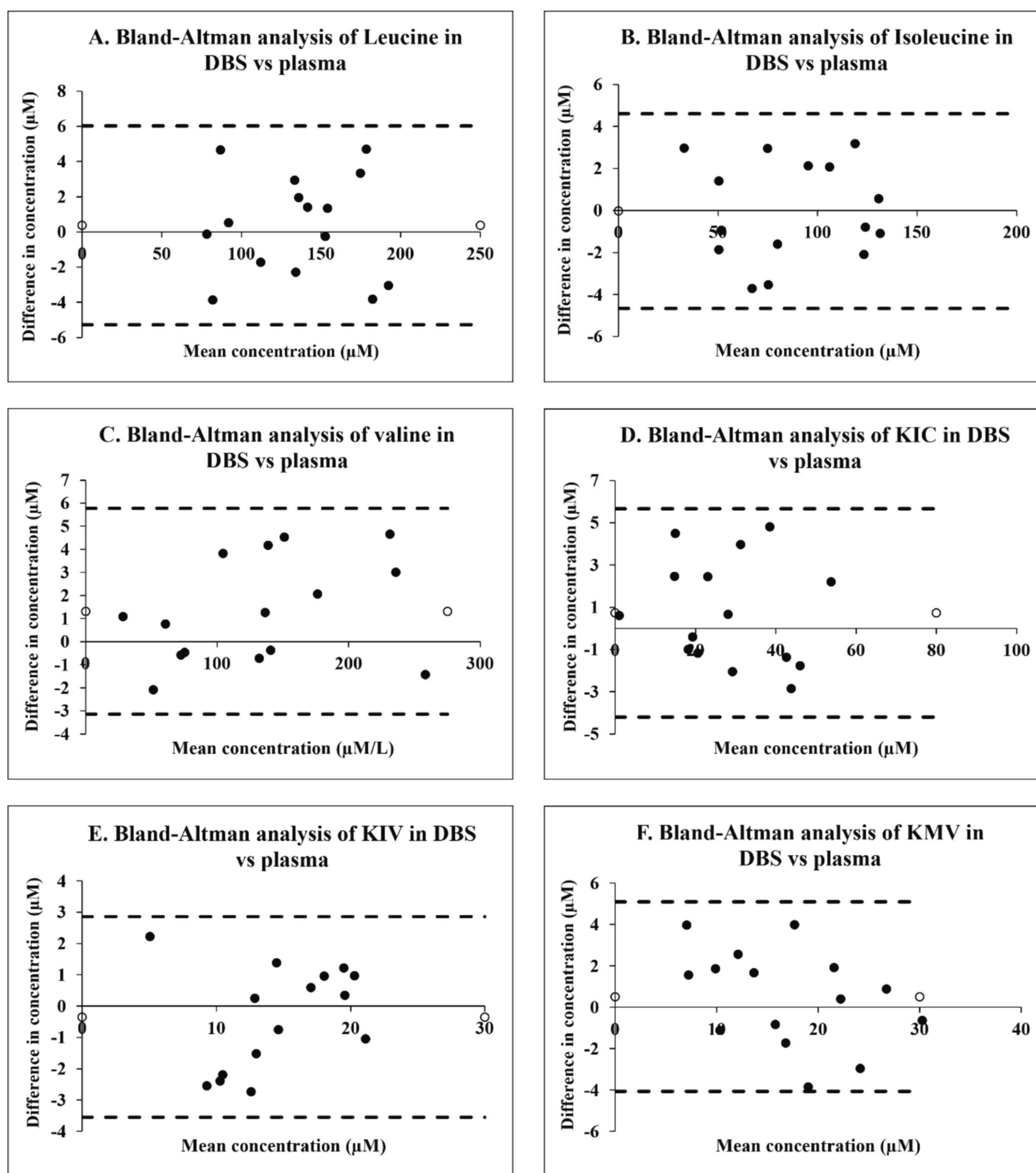

Fig. 7. Bland–Altman difference plot between DBS and plasma method for (A) Leucine, (B) Isoleucine, (C) Valine, (D) KIC, (E) KIV and (F) KMV.

that DBS serves as a reliable and accurate alternative for measuring plasma concentrations of BCAAs and BCKAs. This is significant, particularly for scenarios where traditional venous blood collection presents challenges, or is impractical. Neonatal populations, for instance, would benefit from this non-invasive approach.

Determination of reference ranges for the biomarkers in population of Udupi district of South India

Reference ranges for analytes can vary significantly across different geographical regions [36,37]. This study aimed to establish reference ranges for biomarkers indicative of MSUD in the neonatal population of Udupi, a coastal district in South India. The study cohort consisted of 51.14 % neonates assigned male at birth and 48.86 % neonates assigned female at birth. The reference ranges for leucine, isoleucine, valine, KIC, KIV and KMV were found to be 48.28–210.83, 18.28 – 129.44, 69.18 – 362.43, 6.22 – 12.80, 7.27 – 16.53 and 14.43–29.62 µM, respectively. Detailed data is available in Supplementary Table S8.

Intriguingly, the reference ranges we identified diverge considerably from those reported in other geographical regions. For instance, a study (n = 120) conducted in Tamil Nadu, India [38] documented reference ranges for leucine and valine in neonatal DBS that partially overlap with our findings (Leu: 33.52–270.00 µM; Val: 31.86 – 265.03 µM). In the Czech Republic, reference ranges in neonatal DBS were reported as 103.7–168.3 µM for leucine, 36.9–64.1 µM for isoleucine, 123.4–208.4 µM for valine, 6.5–12.3 µM for KIV, 16.3–28.7 µM for KIC, and 8.7–16.7 µM for KMV in a cohort of 80 neonates [30]. Additionally, an American study by the Mayo Clinic reported reference ranges of 45–214 µM for leucine, 13–135 µM for isoleucine, and 61–325 µM for valine in neonatal DBS [31].

These variations underscore the importance of establishing localized reference ranges for biomarkers, as they can be influenced by genetic,

environmental, dietary, and socio-economic factors unique to each population. The data from Udupi is particularly valuable for improving the accuracy of diagnostic criteria and treatment protocols for MSUD deficiency in this specific region. Such region-specific reference ranges enhance the precision of clinical assessments, ensuring that healthcare interventions are appropriately tailored to the population being served. This is especially critical in neonatal care, where early detection and intervention can significantly impact long-term health outcomes. The reference range helps in proper diagnosis of the condition, thereby helping in the development of treatment strategies.

Treatment strategies for MSUD primarily focus on dietary management, aiming to restrict the intake of BCAAs while ensuring adequate nutrition for growth and development [39,40]. BCAA-free medical foods and judicious supplementation with isoleucine and valine may also be necessary. In addition to dietary management, close monitoring of MSUD is essential to prevent metabolic crises. For rapidly growing infants, weekly or twice-weekly amino acid profile assessment is essential to ensure adequate nutrition and prevent metabolic crises [2]. This monitoring involves regular assessment of blood amino acid levels, as well as monitoring of other biomarkers such as lactate, pyruvate, and alanine [41,42]. These biomarkers can provide valuable insights into the metabolic status of individuals with MSUD and guide treatment adjustments. At least monthly visits with a metabolic specialist are recommended during infancy to closely monitor the infant's development, dietary adjustment, and address any emerging concerns [2]. Developmental milestones should be assessed at each visit or as needed to monitor the infant's cognitive, motor, and social development. In children, adolescents, and adults, weekly amino acid profile assessment is recommended to maintain optimal metabolic control and prevent complications. Regular visits with a metabolic specialist are essential throughout childhood, adolescence, and adulthood for ongoing management, dietary counselling, and monitoring of growth and development. Routine monitoring of calcium, magnesium, zinc, folate, selenium, and omega-3 fatty acid levels should be conducted to ensure adequate intake of these essential nutrients [43]. The frequency of monitoring and assessment may vary depending on individual factors such as age, metabolic control, and adherence to dietary management. Individuals with MSUD should have an emergency plan in place to manage potential metabolic crises, including access to emergency medical care and appropriate medications. Regular collaboration among healthcare providers, such as metabolic specialists, dieticians, and developmental specialists, is essential for comprehensive care and optimal management of MSUD throughout life.

Greenness of analytical method

The rigorous evaluation of the developed method using the AGREE analytical greenness metrics approach yielded a commendable score of 0.64, reflecting its eco-friendliness and adherence to green chemistry principles. A pictogram (Supplementary Figure S4) visually represents the method's green credentials. Our method stands out for its environmentally advantageous features, notably eliminating the need for acidic components in the mobile phase, which reduces hazardous waste. The comprehensive analytical greenness report, detailed in the Supplementary Materials, provides an in-depth overview of its environmental performance. Overall, the results demonstrate that the developed method is more environmentally sustainable than traditional approaches, offering significant potential to reduce the environmental footprint in analytical chemistry practices.

Significance of the non-derivatized LC-MS/MS method for high-throughput screening and its potential for automation

This assay avoids the complexity and time-consuming steps associated with traditional derivatization methods, streamlining sample preparation and reducing potential sources of error. The non-derivatized approach not only simplifies the process but also enhances throughput, enabling more rapid analysis with greater clinical applicability. Although automation was not specifically tested in this study, the streamlined workflow and minimal sample preparation suggest that this method could be adapted for large-scale screening programs, such as newborn screening for MSUD. The use of DBS instead of plasma further highlights its utility by reducing the need for invasive procedures, particularly in neonates. This assay offers high sensitivity and accuracy, representing a significant advancement in bioanalytical techniques for inborn errors of metabolism (IEM).

Limitations of the study

The study successfully developed a non-derivatized LC-MS/MS method for quantifying BCAAs and BCKAs from DBS samples, but certain limitations should be noted. L-alloisoleucine, a key diagnostic marker for MSUD, was excluded to simplify the assay for broader screening purposes; however, future research could incorporate this marker to enhance diagnostic accuracy. Additionally, although the method's simplicity suggests potential for automation, this was not tested in the current study, and further efforts are needed to adapt the method for high-throughput workflows. The reference ranges were established using a small, region-specific cohort, which may limit generalizability to other populations. Furthermore, while analyte stability was confirmed under specific conditions, real-world storage factors, such as humidity and temperature variability, were not thoroughly examined, warranting further investigation. Finally, additional validation is necessary to ensure the method's accuracy in a variety of clinical settings.

Conclusion

We developed and validated an LC-MS-based bioanalytical method for the simultaneous detection of BCAAs and BCKAs from the DBS samples. The method exhibited both linearity and sensitivity for the biomarkers with an LLOQ of 2 μM for BCAAs and 1 μM for BCKAs. The method linearity range is from 2 to 500 μM for BCAAs and 1 to 250 μM for BCKAs. All six analytes were stable in plasma and DBS matrices at all tested conditions. The method was successfully applied to determine and report the reference ranges for these biomarkers in the population of a coastal district of South India. Substitution of DBS for plasma-based techniques reduces the need for uncomfortable and invasive venipuncture blood samples in newborns. The validated method can be readily transferred to any laboratory having accessibility to LC-MS for the detection of MSUD.

Ethics statement

The institutional ethics committee of Kasturba Medical College, Manipal, granted approval for this study (MUEC/010/2017 dated 08.05.2017, MAHE EC/Renewal-02/2018 dated 10.05.2018, and IEC 303/2019 dated 15.05.2019). Blood sampling from neonates was done by certified medical professionals 48 h after birth in Kasturba hospital, Manipal, India. Informed consent from parents was obtained before sampling.

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CRediT authorship contribution statement

Arya Raveendran: Writing – original draft, Project administration,

Methodology, Formal analysis, Data curation, Conceptualization. **Ashutosh Gupta:** Writing – original draft, Formal analysis, Data curation, Conceptualization. **Leslie E. Lewis:** Writing – review & editing, Visualization, Supervision, Resources, Investigation, Conceptualization. **Krishnananda Prabhu:** Writing – review & editing, Visualization, Supervision, Resources, Investigation. **Sudheer Moorkoth:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmsacl.2024.10.001.

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